Cryopreservation of Adipose tissue and effect of 17β-estradiol on Osteogenesis of Omental fat derived stem cells

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Technology

In

Biotechnology

By

Shaik Mulla Shahensha

(Reg. No. 210BM2018)

Department of Biotechnology and Medical Engineering

National Institute of Technology, Rourkela

Rourkela-769008

(2010-2012)

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Under the Guidance of

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S M SHAHENSHA

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Contents

Abstract:

Due to the wide range of applications in reconstructive surgeries and in tissue engineering, the urge to cryopreserve adipose tissue has increased. Cryoprotectants are known to protect the cells from cryoinjury. Investigators across the world are trying to find out ideal cryoprotectants and to develop optimum cryopreservation protocol. However, limited success has obtained yet.

This study is a small endeavor to determine the effect various sugars on fat preservation. The cryoprotective action of Mannitol, Sucrose, Trehalose, DMSO, Glycerol, PBS and normal saline have studied. The cryoprotective solutions are prepared in various concentrations and added to fat samples and plunged into liquid nitrogen. The cell viability after post thawing has been evaluated by fat oil ratio and Glycerol 3 phosphate dehydrogenase enzyme activity assay. The results indicated that 5% trehalose, 10% DMSO has shown better cryoprotective action than others. Mannitol has shown the least cryoprotective action in comparison to all the cryoprotectants used in the study. Interestingly, it was found that fat preserved in PBS has good cell viability next to 5%trehalose and 10%DMSO.

Omental fat is rich in factors that promote the healing and regeneration. The effect of 17-β-estradiol on the omental adipose derived stem cells during osteogenic differential potential has been studied. The results of the study indicated that 17-β-estradiol enhances the osteogenic differentiation of omental fat derived stem cells.

Keywords: Cryoprotectants, cryoinjury, Omental fat, 17- β –estradiol.

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1. Introduction

Autologous fat transplantation (aft) is commonly used in reconstructive plastic surgeries to rejuvenate hands, face and other body parts. The abundant availability of adipose tissue in body, ease of harvesting makes adipose tissue a best filler in Autologous fat transplantation[1]. The major limitation of aft is the high rate of absorption of transplanted tissue and often requires repeated corrections [2-4]. Surgeries are often required causing patient discomfort. The good solution to this problem can be collecting extra tissue during the first surgery and preserving it for future use[5]. Not only adipose tissue cryopreservation is helpful for those patients who in need of the fat transplantations but a successful adipose tissue cryopreservation protocol may lead to isolation of stem cells from it in future. Adipose tissue derived stem cells (ADSCs) are the mesenchymal stem cells with huge differential capability towards different cell lineages like adipogenic, chondrogenic, osteogenic, cardiogenic, neurogenic and hepatogenic. Because of its capability, it has huge clinical potential as cellular and tissue engineering therapy. To cater these huge clinical need cryopreservation of ADSCs is necessary. A successful cryobank will maintains the clinical quality cell in a standardized environments and will be used during the need. But cryo bank of ADSCs has its own limitations. The cells need to cultured from the harvested adipose tissue which is time consuming and costly process. Another problem associated with successful cryobank is lack of proper characterizations of the ADSCs and lack of cost effective proliferative agents. The objectives of our study are to overcome these limitations. In the first objective, we have tried to cryopreserve the adipose tissue using different cryoprotectants so that adipocytes remain viable and ADSCs can be retrived back in future. The second objective deals with the characterizations of ADSCs and use of estradiol as the proliferative agent and differentiating factor for ADSCs.

2. Literature review

2.1 Basic principles of cryopreservation

Cryopreservation is a long-term preservation technique and it can be defined as the use of very low temperatures (typically colder than -130°C) to preserve structurally intact living cells and tissues. Effect of solute concentration[6], rate of cooling and intracellular freezing[7], cell packing effect[8] are the major problems associated with the cryopreservation.

The growth of ice crystals in extracellular medium during freezing increases the solute concentration resulting in osmotic stress on the cells[6]. By his experiments on haemolysis of Red blood cells, J. E. Lovelock[6] describes that it is the increase in solute concentration during extracelluar ice formation which causes the freezing injury rather than the extracellular ice itself.

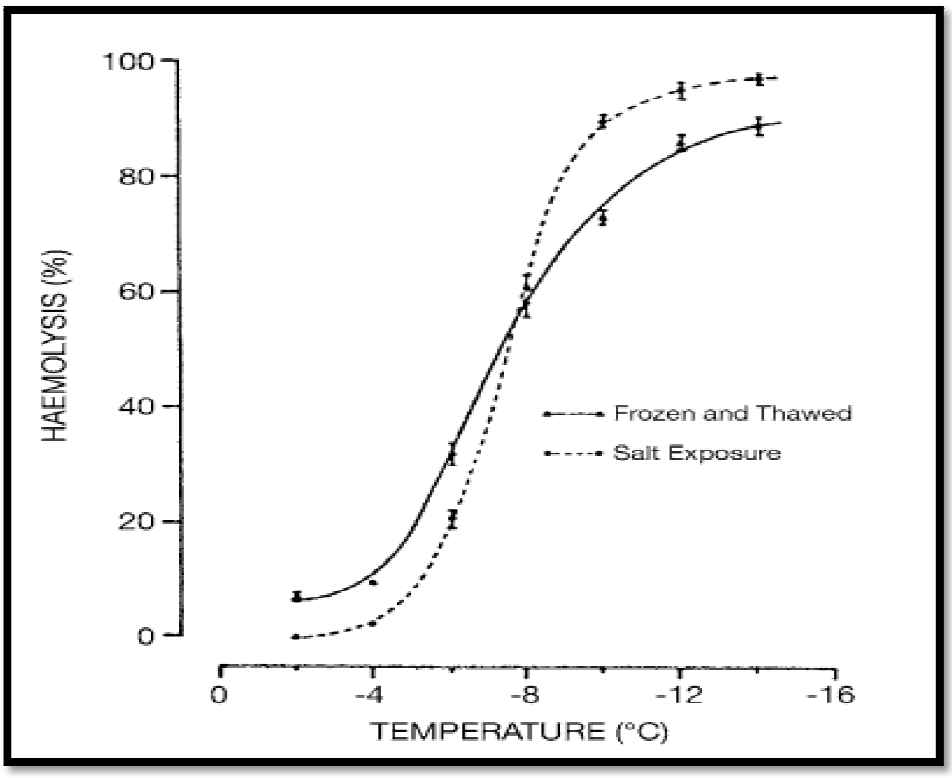


Figure 1: Lovelock experiment shows the increase in haemolysis of Red blood cells by exposure to salt[9]

The formation of intracellular ice has deleterious effect on the cell[10]. Organelle disruption, physical destruction of membranes and gas bubble formation may be the possible mechanisms of cell damage by intracelluar ice[11].

The findings of P. Mazur[7] revealed that the rate of cooling during freezing has a vital role in controlling the water transport across the cell membrane as well as intracellular ice formation. Slow cooling rate aids the formation of extracellular ice; hence increasing the solute concentration and thus resulting osmotic stress on the cells.

In case of rapid cooling rate, the cytoplasm will become supercooled and there will be less chance for water to elute out, increasing the proability intracellular freezing. The effect of slow cooling rate will be the loss of water from the cell causing the cell shrinkage (fig.1). In addition, slow cooling rate induce the extracellular ice formation trigerring the solute concentration effect on the cell. Hence, an optimal cooling rate has to be choosen based on the above mentioned factors.

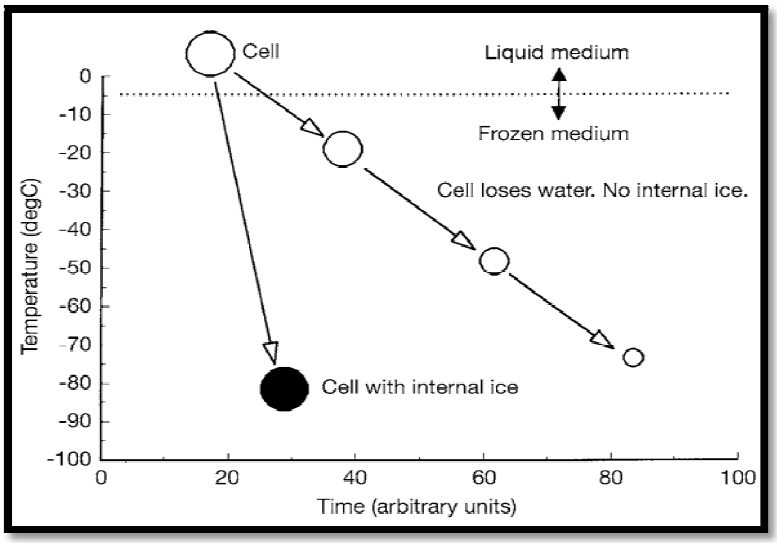


Figure 2: The possible mechanisms of effect of rapid cooling rate and slow cooling rates on cells. (based on ref [12])

Not all the cells have same cooling rate. The optimum cooling rate vary from cell to cell depending on their source (Fig. 3). The combination of proper cyroprotectant with optimal cooling rate will enhance the cell recovery after the preservation process[8].

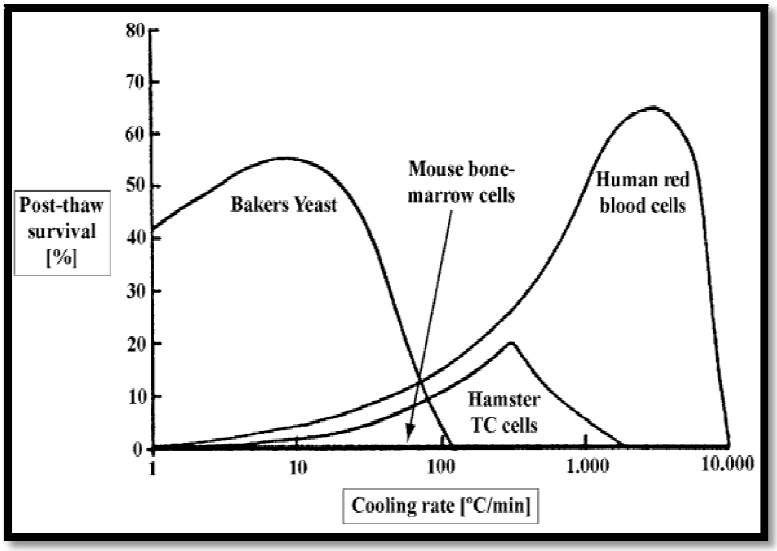


Figure 3: The effect of cooling rate on post-thaw recovery of cells from different sources [12]

The addition of cryoprotectants will reduce the lethal effects associated with ice formation. The ideal cryoprotectant should be highly soluble in water; able to penetrate into the cells and must have a low toxicity[12].

Cell packing effect: Most studies of freezing injury have been carried out with relatively dilute cell suspensions, whereas the cells are quite densely packed in some systems that have to be reserved, for example red blood cells for transfusion, and particularly in tissues and organs. Experiment has shown that the proportion of red blood cells suspended in 2.5 M glycerol solution that are hemolyzed during freezing and thawing is strongly dependent on hematocrit (the percentage of cells by volume) when the hematocrit exceeds 50%. The increase in hemolysis as the hematocrit is increased is ameliorated by increasing the glycerol concentration. At 2 M glycerol concentration, hemolysis is inversely dependent on warming rate when the cooling rate is less than 1000 ° C/min and is directly dependent on cooling rates at higher cooling rates (11).

These observations cannot be accounted for by the classical mechanisms of cryoinjury solution effects and intracellular freezing. The most likely explanation is that densely packed cells are more likely to be damaged by mechanical stresses when the channels within which they are sequestered change shape as a result of recrystallization of the ice that form their boundaries.

Mechanism of cryoprotective agents (CPA): Glycerol is used as a cryoprotectant to preserve spermatozoa for the first time by polge and collegues[13]. The works of Polge et.al, has paved a way for the development of cryopreservation techniques. As glycerol is a poly-hydroxylated solute, it can form hydrogen bonds with water and can permeate the limiting plasma membrane. The cells can tolerate the glycerol concentration upto 5 mol/l. However, it completely depends upon cell type and exposure conditions. By considering these properties, Lovelock has proposed a theory of colligative action[14]. According to his theory, the depression in freezing point is associated with mixtures of solutes in solution and at any given temperature below the ice transition during cooling, the rise in salts (as sodium chloride is main component in most of media) would be ameliorated by the presence of glycerol. The increase in high viscosity of glycerol during lowering of the temperature is may inhibit ice crystal growth.

Solutes ranging from sugars, alcohols, amides to diols have been investigated for cryoprotective activity during past two decades [15-16]. However, majority of them have not found their way in modern cryopreservation protocols due to relative lack of cryoprotecting efficiency. Solutes such as dimethyl sulphoxide, trehalose, sucrose, 1,2 propanediol and ethanediol have shown high cryoprotecting activity. DMSO interacts electrostatically with phospholipid bilayers[17]. Similarly, small molecular weight solutes glucose, lactose, ribose, alanine, glycine, proline and amides including acetamide and formamide posses cryoprotecing activity but at a low efficiency.

Solutes may interact with the proteins either by preferential binding or preferential exclusion from the surface. Preferentially excluded solutes may stabilize the proteins thermodynamically under conditions like dehydration during freezing. Disaccharides such as sucrose and trehalose stabilize the membranes by interacting with polar head groups of phospholipids[18].

The cryoprotection activity of high molecular weight compound like polymers has exposed by the experiments done by Bricka and Bessis[19]. They demonstrated the cryoprotective action of polyvinylpyrrolidone and dextran on human erythrocytes. Hydroxyl ethyl starch is also used as cryoprotectant to preserve erythrocytes by Doebbler & Rinfret[20]. Sputtek and colleagues used hydroxyl ethyl starch in freezing erythrocytes

and produced a systematic study for autologous transfusion. The cryoprotective action of high molecular weight solutes is found to be effective to preserve erythrocytes. However, high molecular weight solutes have shown limited efficacy in preserving nucleated cells[21]. The presence of large number of hydrophilic side chains in these polymers increases the probability of hydrogen bonding resulting in the increase in viscosity during freezing. This increase in viscosity may help in retarding the ice crystal formation and these agents are also known to suppress ice nuclei in the extracellular spaces[22]. Relatively high cooling rates usually -200 and -500 ˚C/min are required to obtain best results[23].

The toxicity nature of CPAs are highly complex to understand. It depends on the exposure to high concentrations of salts, time and temperature-dependent in most cases. To avoid these effects, attempts have been made by using lower temperatures of exposure and by minimizing the exposure time to CPA before and after freezing. However, lowering exposure temperatures may decrease the passive permeation of the CPA into cells and thus increasing the time to achieve the sufficient concentrations. Fahy[24] reviewed that there might be an underlying chemical toxicity of DMSO, despite of reducing the effect of salt concentration by its colligative action.

2.2 Adipose tissue cryopreservation

Excess fat grafts harvested during plastic and cosmetic surgeries are generally discarded[25]. The potential applications of Adipose tissue, filler during cosmetic surgeries[1] and source of mesenchymal stem cells[26] have urged the need to long term preservation the adipose tissue. Preserving the tissue in intact form maintaining the high cell viability and recovery rate are the challenging tasks. The results obtained by freezing the aspirates at temperatures (+1ºC to -18ºC) were unsatisfactory for long term preservation[27].

Several investigators across the world are trying to develop an optimum method for long-term preservation of adipose tissue using cryopreservation [28-32]. B. Atik*, et al.*, [5] demonstrated that the fat grafts freezed in liquid nitrogen have the similar cell viability in comparision to fresh fat tissue. L. L. Pu*, et al.*,[28] used a combination of (Dimethyl sulphoxide) DMSO and Trehalose as cryoprotectants to preserve adipose aspirates by cryopreservation method. DMSO is an effective cryoprotectant but due to its toxicity it has to be removed from cells before clinical use[8]. So, to determine the role of trehalose in cryopreservation, L. L. Pu*, et al.*,[32] have used trehalose as sole cryoprotectant to preserve adipose aspirates. Their results with trehalose as cryoprotectant suggest that it can improve the long term presevation of adipose aspirates and further investigation has to be done to develop optimal protocol for cryopreservation. The results of Wolter T. P, *et al.*, [31] indicated that the tissues preserved by the addition of cryoprotectants have more cell viability than the tissues that were preserved by normal freezing process. The study conducted by D. Son*, et al.*,[33] revealed that the current preservation techniques of storing the adipose tissue at temperatures -15ºC and -80ºC are insufficient to maintain the cell viability in the adipose tissue. J. W. MacRae*, et al.*,[29] observed the improved mitochondrial activity in the fat preserved at subzero temperatures as compared with fat stored at 32 ºC. The experimental studies of shoshani et al., [34]described that fat stored at -18 ºC for 2 weeks also has viability. The *in vivo* studies Rinker et al.,[35] demonstrate that the long-term survival of cryopreserved composite tissue transplants are feasible and support an indirect injury, rather than direct injury from freezing or cryoprotectant agents. Survival of aspirated fat cell grafts for autologous fat transplantation mainly depends on

the anatomic site, the mobility and vascularity of the recipient tissue, or underlying causes and diseases, and less on harvesting and reinjection methods[36]. The experimental results of Ullmann et al., [37] suggests that fat frozen for more than 7 months will have less cell recovery. Narins et al., [38] demonstrated that fat frozen is safe for autologous fat transplantation. However, the experiments from Moscatello et al., [30] revealed that fat preserved without addition of cryoprotectant will not be viable and it cannot be useful as a filler. If preserved at room temperature, the aspirated fat has to be transplanted as quickly as possible[39]. Luyet et al.,[15] has compared the efficacy of ethylene glycol, glucose and sodium chloride in protecting the tissues during freezing.

It was reported that plant seeds, fungal spores, yeast cells and some nematodes, tardigrades and rotifiers evolved a strategy of secreting certain substances which protect them in freezing environments[40]. *Aphelenchus avenae,* a nematode, converts 20% of its dry weight to trehalose in order to combat with dehydration[41]. *Pseudomonas* species release some compounds in their medium, which reduces the nucleation temperature of water. The seeds extracts of apricot, plum and peach reduce the nucleation temperature of water droplets[42]. The gallfly *Eurosta solidaginis* contains ice-nucleating agents in its haemolymph which makes it freeze tolerant[43]. To survive in extreme cold environments, some insects synthesize polyols[44]. All the above examples reveal that these organisms have adapted to adverse environmental conditions and evolved a strategy of secreting special substances known as cryoprotectants to protect themselves from such adverse environmental changes. Extensive research on the cryoprotectants and their application will revolutionize the field of cryobiology.

2.3 Adipose derived stem cells:

Progressive osseosis hetroplasia is a genetic disorder, which results in formation ectopic bone in subcutaneous adipose layer of skin in children. The presence of adipocytes, chondrocytes and osteoblasts were found in histological examination of those tissues[45]. Thus, the pathology examination reveals the presence of stem cells in the adipose tissue. The mature adipocyte is terminally differentiated and cannot proliferate further[46]. By using radioactive tracing it was found that the turnover rate of cells in adipose tissue ranges between 6to 15months [47-48]. The existence of stem cell population within adipose tissue is responsible for replacing mature adipocytes through the lifetime of the individual.

The isolation of mesenchymal stem cells from adipose tissue is first demonstrated by Zuk et al.,[26]. The potential of adipose tissue derived stem cells into adipogenic, chondrogenic and osteogenic has also been demonstrated by zuk and colleages. Later many researchers have successfully isolated and differentiated the adipose tissue derived cells into various cell lineages. Adipose tissue is an abundant source of stem cells and is the best alternative to the bone marrow derived stem cells. The frequency of obtaining stem cells bone marrow is 1 in 100000 whereas from adipose tissue is 1in 100 which is relatively high. The adipose tissue is abundantly available in body, which can be easily harvested whereas the bone marrow isolation is a painful procedure for the patient.

With their therapeutic applicability in the fields of tissue engineering and regenerative medicine, ADSCs make themselves as better alternative to bone marrow derived stem cells. As per the criteria defined by International society for cellular society a mesenchymal stem cell should be plastic adherent, differentiate into adipogenic, osteogenic and chondrogenic cell lineages and must express the surface markers CD90, CD105, CD73 and the expression of surface markers HLA-DR, CD 34, CD45, CD14 or CD11b, CD79a or CD19[49]. However, the expression of CD34 is shown in the ADSCs during early passages[50]. The differential potential of ADSCs is not limited to adipogenic, chondrogenic and osteogenic but they can also be differentiated into several other lineages such as hepatogeneic, neurogenic and Cardiogenic. Ashjian et al., [51] have differentiated the ADSCs into early neural progenitor cells by treating them with

5µg/ml insulin, 200µM indomethacin, 0.5mM isobutylmethylxanthine. Seo et al.,[52] demonstrated the differentiation of ADSCs into hepatocytes by treating them with 0.1% DMSO, 10 ng/ml rhOSM and rhHGF in a serum free medium for 2-3 weeks. The differentiation of ADSCs into cardiomyoctes was demonstrated by Rangappa et al.,[53]. By treating the ADSCs with 5-azacytidine Rangappa et al., have achieved the cardiogenic differentiation.

The expression profile of ADSCs varies from passage to passage. Immunophenotypic studies on human adipose derived stem cells done by Mitchell et al., [54] reveals the following. The low expression of stromal cell associated markers CD13, CD29, CD44, CD63, CD73, CD90, CD166 were found during initial passages. However, significant increase in the expression of surface markers was observed with successive passages. During the initial passages, the expression of CD34 was at peak levels in the stromal vascular fraction cells (SVF) although decrease in expression of CD34 was found, throughout the culture period. Aldehyde dehydrogenase and the multidrug-resistance transport protein (ABCG2) were expressed by SVF cells and ASDCs at detectable levels. The expression of Endothelial cell-associated markers, CD31, CD144 or VE-cadherin, vascular endothelial growth factor receptor 2, von Willebrand factor, on SVF cells present throughout the culture.

The surface antigen expression of ADSCs is a controversial topic. The Positive expression of stem cell associated market STRO-1 ADSCs is reported by Zuk et al., [55] whereas the lack of expression of STRO-1 on ADSCs is reported by Gronthas et al [56]. Zuk et al., also reported that the expression of two antigenic markers CD49d and CD106 differs between ADSCs and other mesenchymal stem cells (MSCs). The expression of CD49d is predominant in ADSCs and there is no expression of this marker on other MSCs. ADSCs does not expressed CD106 whereas its expression was found on other MSCs.

Gronthas et al., reported that expression of surface antigens CD9, CD10, CD13, CD29, CD34, CD44, CD49e, CD54, CD55, CD59, CD105, and CD166 was present on undifferentiated ADSCs.

2.4 Omental fat:

The omentum fat is attached to the greater curvature of the stomach lining the peritoneum[57] acting as the omental pedicle to injured organs[58].

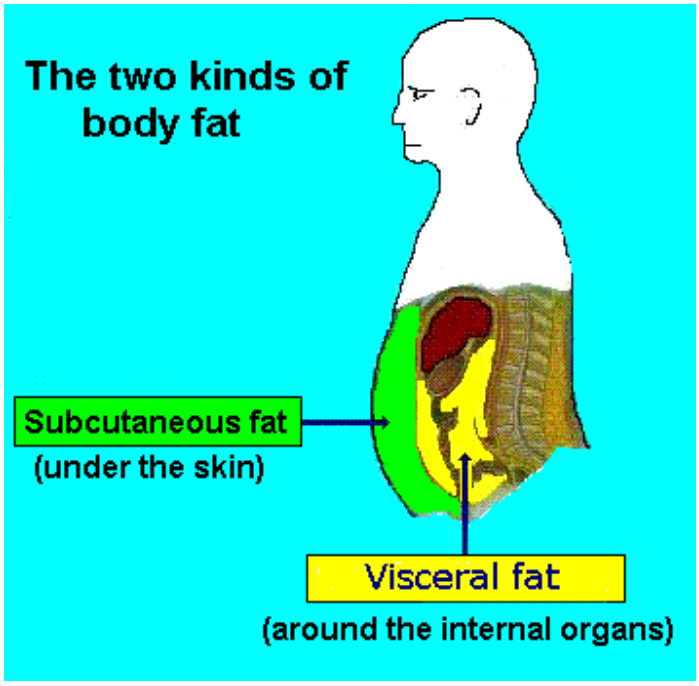


Figure 4: Representation of omental fat and subcutaneous fat

Omental fat promotes the healing and regeneration of neurons across a transected spinal cord [59] resulting in the significant recovery of limb function. The stromal cells isolated from omental fat expresses the adult stem cell markers (SDF-1 α, CXCR4, WT-1) and embryonic pluripotent markers (Oct-4, Nanog, SSEA-1)[60]. The experiments of Singh et al.,[60] revealed that the omental stem cells migrate to injured sites and enhance the healing process. Singh et al., labeled omental cells and injected in the surrounding of subcutaneous granulation tissue and after 24h the migration and integration of labeled omental cells into the growing granulation tissue was observed. The experiments by Marapogoundar et al.,[61] revealed that subcutaneous fat derived stromal cells showed the side population epression of ABCG2, whereas no ABCG2 expression is found in omentum fat.

2.5 17β estradiol:

17β-estradiol, is a sex hormone and has 2 hydroxyl groups in its molecular structure[62].

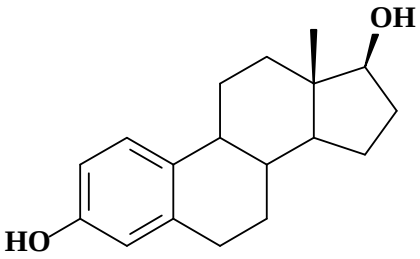


Figure 5: Structure of 17β-estradiol having two hydroxyl groups. (Drawn using chemwindow software)

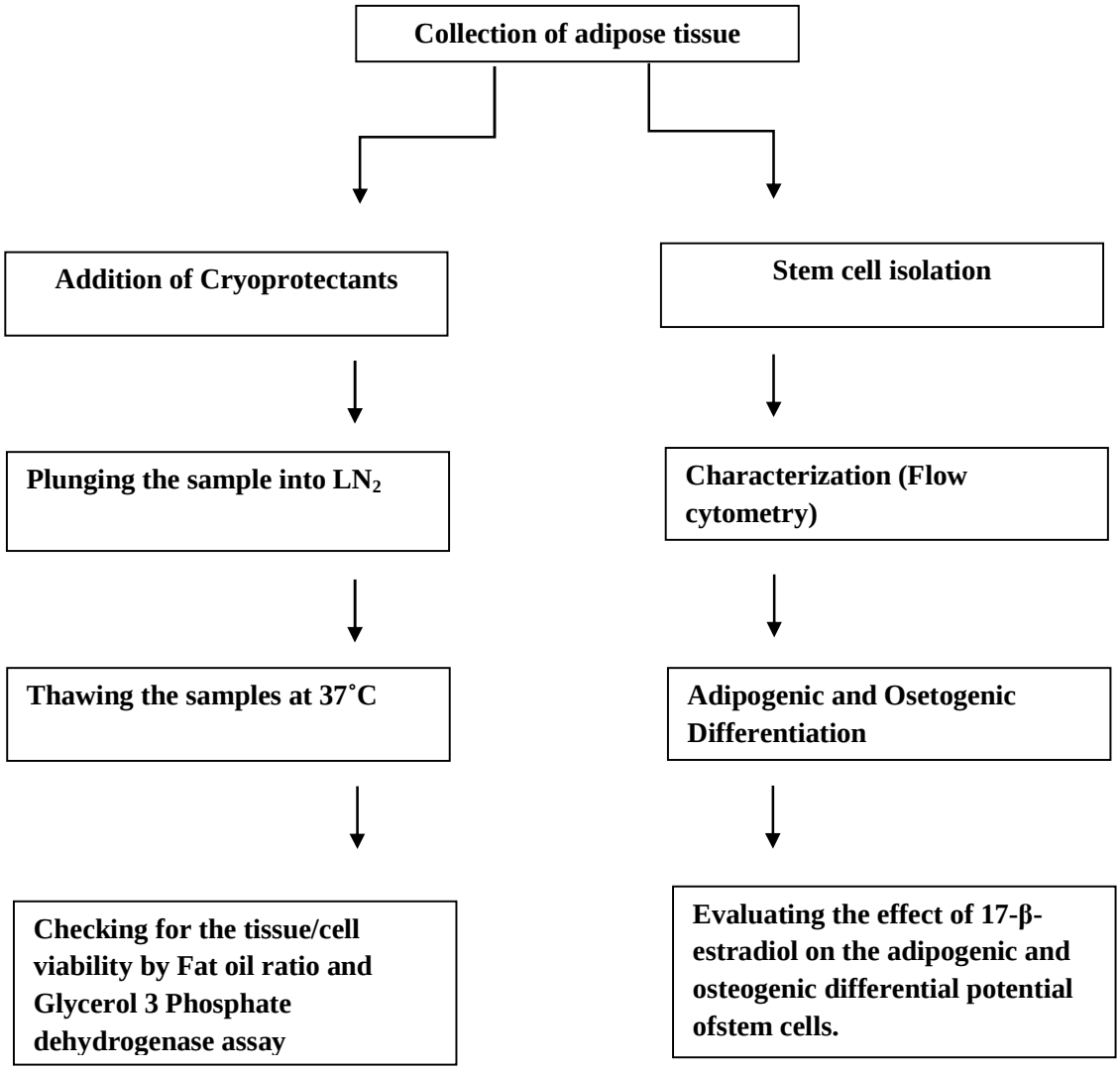
It is also called as Estradiol (E2). In females, the serum levels of E2 depend on the menstrual cycle. The concentration varies from 19 to 140 pg/ml. In postmenopausal women, the serum level of estradiol drops to less than 35 pg/ml[63]. In males, estradiol is produced as an active metabolic product of testosterone and the serum levels of estradiol in males vary from 14-55 pg/ml. Estrogen depletion in postmenopausal women leads osteoporosis which is characterized by bone loss and increase in bone fragility[64].

It was reported that 17-β-estradiol improves the osteogenic and adipogenic differential potential of human bone marrow MSCs and neural differentiation of embryonic stem cells[65]. Liu hong et al., reported that 17-β-estradiol enhances osteogenic and adipogenic differentiation of human subcutaneous adipose derived stem cells (ADSCs)[66]. However, the effect of 17-β-estradiol on the human omental adipose derived stem cells is unknown. Moreover, the effects of 17-β-estradiol on the different genders and different ages have not completely studied.

3. Objective

- To preserve the adipose tissue by maintaining the high cell viability using various cryoprotectants.
- To study the effect of 17-β-estardiol on osteogenic and adipogenic differential potential of omental adipose derived stem cells.

4. Plan of work



5. Material and methods

5.1 Fat oil ratio: Human omental fat was collected with proper consent from patients undergoing cholecystectomy surgeries from Super specialty hospital, Rourkela. The collected fat sample was thoroughly washed with phosphate buffered saline (PBS). The cryoprotectant solutions of DMSO, Glycerol, Sucrose, Mannitol, and Trehalose in the concentrations of 5%, 10% and 15% V/V, W/V were prepared accordingly in distilled water. PBS and Normal saline solutions were also used in this study. Equal volume of cryoprotectant solution was added to fat and mixed thoroughly. Then, these samples were plunged into liquid nitrogen. After one or 2days, the samples were taken out and thawed at 37 ˚C hot water bath (remi).The thawed fat samples were centrifuged at 200Xg for 15minutes. Later, oil to fat ratio was calculated by using formula.

$$Fat\ Oil\ ratio = Oil/(Fat + Oil)$$

5.2 Glycerol 3 phosphate dehydrogenase (GDPH) enzyme activity assay: After centrifugation, three layers of oil, fat graft and fluid will be formed. GDPH is a strictly intracellular enzyme and is specific to adipocytes. The damaged adipocytes during preservation process release the GDPH enzyme extracellularly. The fluid portion after centrifugation is collected for the assay that contains the enzyme released from the damaged cells. The assay is performed by GDPH assay kit (Takara Bio Inc #MK426). The decrease in the absorbance of NADH by itsconversion to NAD+ in the presence of enzyme GDPH is measured at 340nm in UV spectrophotometer (Systronics). The protocol described by the kit's manufacturer is followed. The enzyme activity is calculated by using the following formula.

$GDPHactivity\left(\frac{Unit}{ml}\right) = (\Delta OD@340nm * A\ (ml) * Dilutionratio)/(6.22 * B\ (ml) * C\ (cm))$

$\Delta OD@340nm = Decrease\ in\ absorbance\ at\ 340\ nm\ per\ minute$

$A(ml) = Total\ reaction\ volume$

$B(ml) = The\ volume\ of\ enzyme\ solution\ added$

$C(cm) = Optical\ path\ length\ of\ the\ cell\ used$

$6.22 = Millimolar\ absorption\ coeffiecient\ of\ NADH\ molecules$

5.3 Stem cell isolation: The omental adipose tissue was collected aseptically from Super specialty Hospital Rourkela. The adipose tissue was washed with PBS and minced. The tissue was digested by addition of 0.075% collagenase(Himedia TC211) and incubated at 37 ˚C for 30-60minutes. The action of collagenase enzyme was neutralized by addition of 10% FBS (Himedia RM9952). Then, the digested tissue was centrifuged at 1200xg for 20min. High density pellet willbe obtained which was treated with 160mM NH_4Cl to lyse the red blood cells and strained through 100µmcell strainer to remove any debris. Thus, obtained solution was again centrifuged and the pellet was suspended in the control media containing DMEM (Himedia AL149A), 10% FBS and 1% anitibiotic/antimycoticsolution(Himedia A002) and incubated in 5% CO_2 incubator at 37 ˚C.

5.4 Characterization by Flow cytometry: Once the confluence is reached, the cells were trypsinised and the concentration of 10^6 cells/ml is maintained. The cells were incubated with the antibodies conjugated with the fluorescent dyes FITC, APC, PerCp, PE etc. After the incubation, the prepared cells samples were analyzed for the expression of mesenchymal stem cell markers CD73, CD105, CD90 as well as the absence of negative markers HLA-DR, CD45 using the flow cytometer (BD Fortessa)

5.5 Osteogenic Differentiation: The osteogenic differentiation is induced by treating the cells with Ascorbic acid (TC099) and β-glycerophosphate (MP Biomedicals., 195206). The osteogenic differentiation media was prepared by adding 10mM concentration of β-glycerophosphate and 300µM concentration of ascorbic acid to DMEM medium containg 10% FBS. The osteogenic differentiation media was changed for every 3 days. After 22 days the cells were stained with alzarin red stain for the detection of osteoblasts under phase contrast inverted microscope (Magnus Invi-TR) and images were captured. The mean percentage area of the stained regions is analyzed by Image J software.

5.6 Quantification of alizarin red stain: The stained regions are dissolved with 10% acetic acid and incubated for 30 minutes followed by 15 minutes incubation at 85 ˚C. This sample was collected and the absorbance is measured at 405nm in UV spectrophotometer.

5.7 Adipogenic Differentiation: The adipogenic differentiation was induced by treating the cells with adipogenic media (Himedia AL502). After 18-20 days, the cells were stained with oil o red for the detection of adipocytes.

5.8 Differentiation in the presence on 17-β-estradiol: The osteogenic and adipogenic differentiations were induced in the presence of 17-β-estradiol (Himedia RM4682). The concentrations of 10nM and 20nM were used and the results were compared with controlsto determine the effect of 17- β-estradiol.

6. Results:

Fat oil ratio: After centrifugation at 200Xg, the fat oil ratio was measured and results obtained with various cryoprotectants were compared.

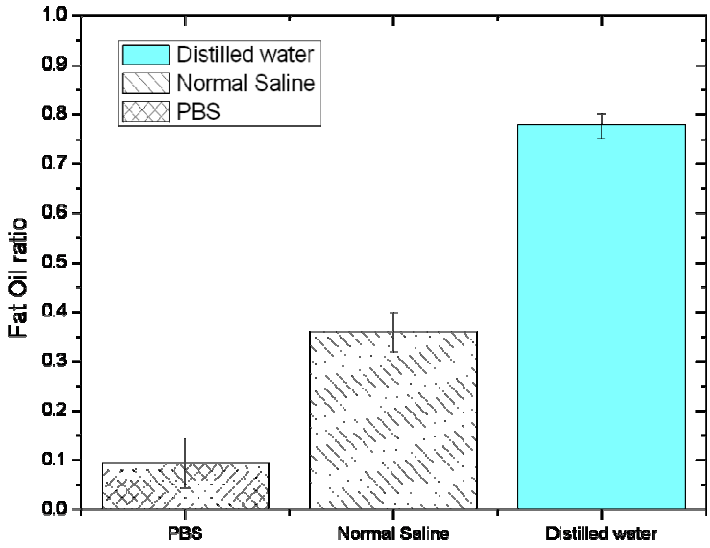


Figure 6A

Figure 6A represents the graph showing the effect of PBS, normal saline and Distilled water during preservation. The fat oil ratio of sample stored in PBS is found to be very less in comparison to normal saline and distilled water. As all the cryoprotecting solutions are prepared in distilled water, sample in distilled water can be stored as control.

Figure 6B

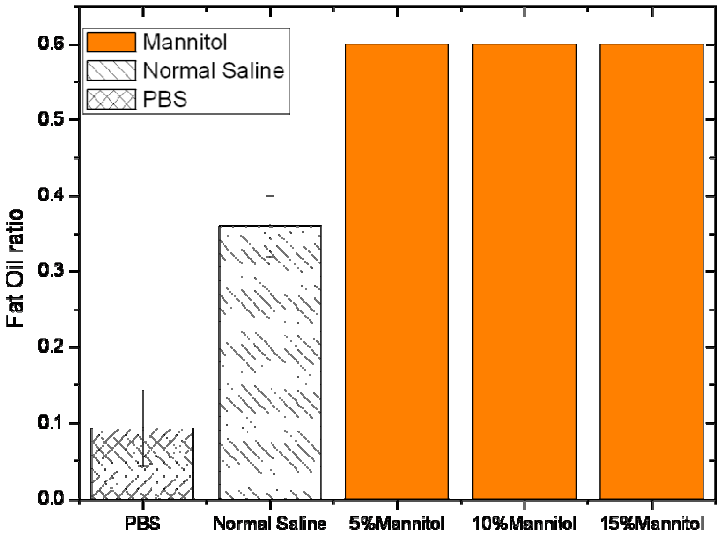


Figure 6B represents the graph showing the effect of PBS, Normal saline and 5%, 10%, 15% Mannitol during preservation. The fat oil ratio of sample stored in PBS is found to be very less in comparison to normal saline and Mannitol.

Figure 6C

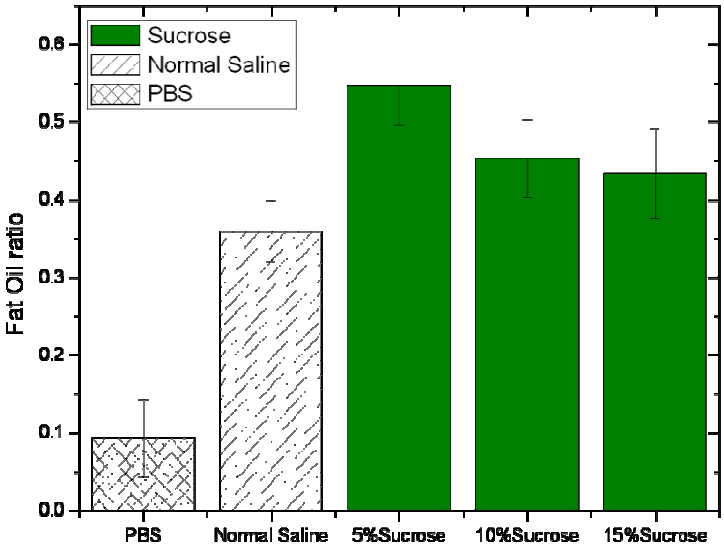


Figure 6C represents the graph showing the effect of PBS, normal saline and 5%, 10%, 15% Sucrose during preservation. The fat oil ratio of sample stored in PBS is found to be very less in comparison to normal saline and Sucrose.

Figure 6D

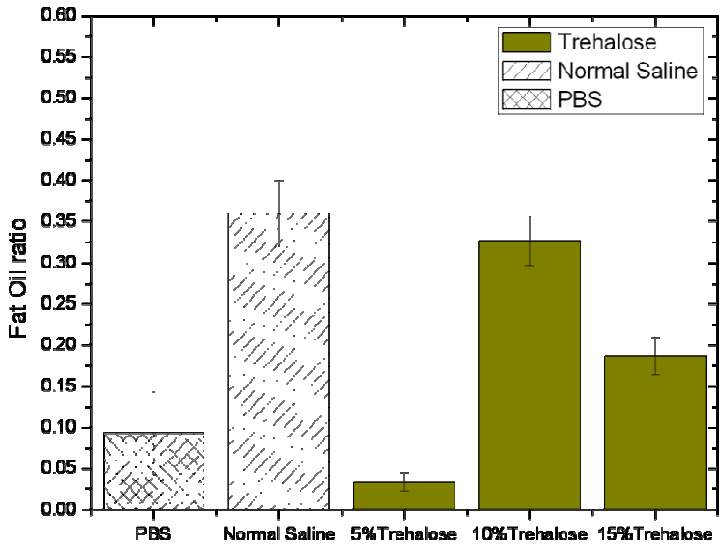


Figure 6D represents the graph showing the effect of PBS, normal saline and 5%, 10%, 15% Trehalose during preservation. The fat oil ratio of sample stored in 5% Trehalose is found to be very less. Sample in PBS has shown better result in comparison to 10% and 15% trehalose.

Figure 6E

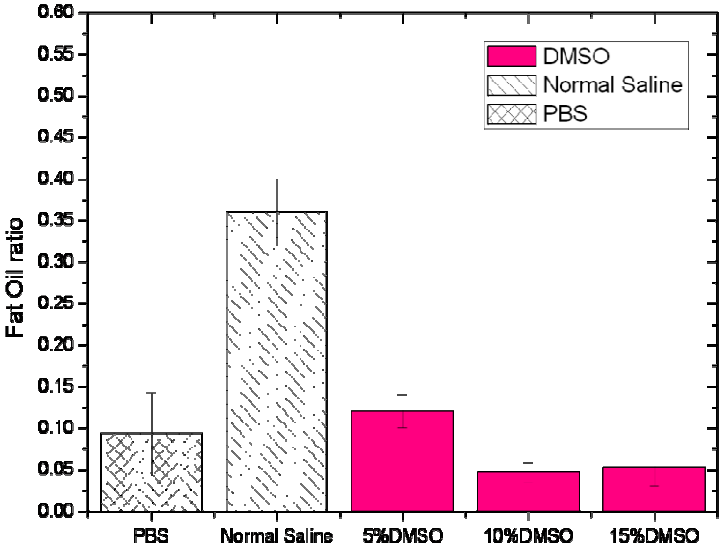


Figure 6E represents the graph showing the effect of PBS, Normal saline and 5%, 10%, 15% DMSO during preservation. The fat oil ratio of sample stored in 10% and 15% DMSO is found to be very less in comparison to 5% DMSO and PBS

Figure 6F

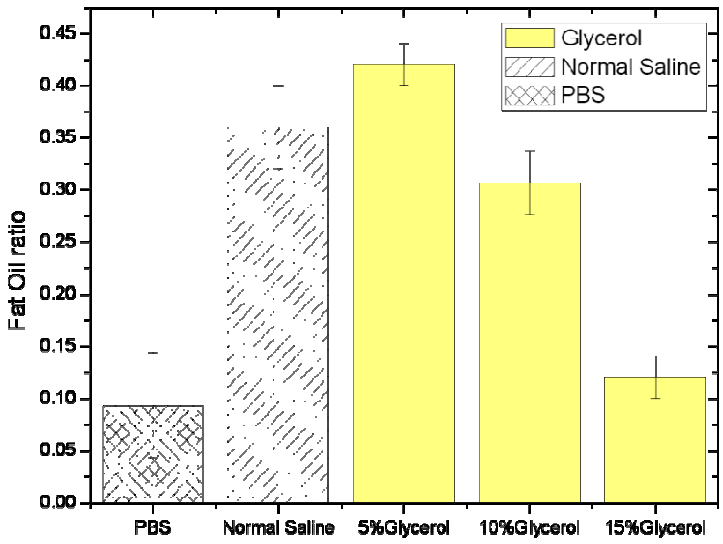


Figure 6F represents the graph showing the effect of PBS, Normal saline and 5%, 10%, 15% Glycerol during preservation. No significant difference was observed between fat oil ratio of samples stored in PBS and 15% glycerol. However, the fat oil ratio was more in comparison to 15% glycerol and PBS.

GDPH enzyme activity assay: The aqueous layer obtained after centrifugation was taken to analyze the amount of GDPH enzyme that was released by damaged cells.

Figure 7A

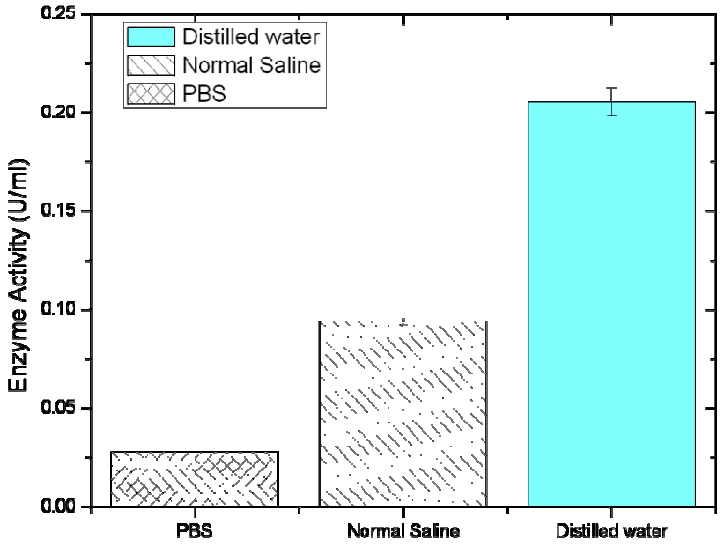


Figure 7A shows the graph of GDPH enzyme activity of samples preserved PBS, normal saline and Distilled water. Sample in PBS has shown less enzyme activity than normal saline and distilled water.

Figure 7B

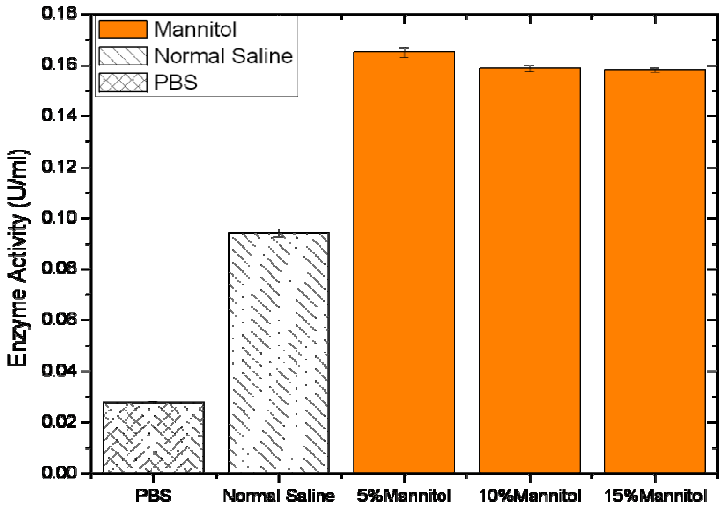


Figure 7B represents the graph of GDPH enzyme activity of samples preserved PBS, normal saline and 5%, 10%, 15% Mannitol. Sample in PBS has shown less enzyme activity in comparison to normal saline and Mannitol. Samples in Mannitol have highest enzyme activity indicating the less cryoprotectant activity.

Figure 7C

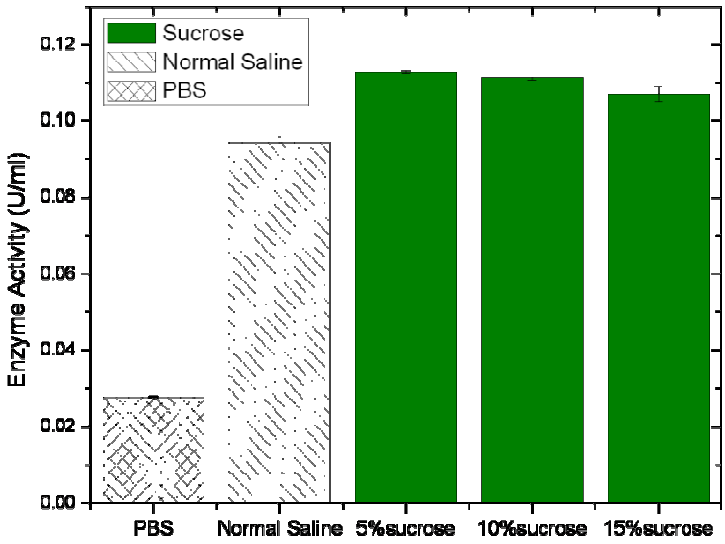


Figure 7C shows the graph of GDPH enzyme activity of samples preserved PBS, normal saline and 5%, 10%, 15% sucrose. Sample in PBS has shown less enzyme activity in comparison to normal saline and sucrose. Samples in sucrose have more enzyme activity than normal saline.

Figure 7D

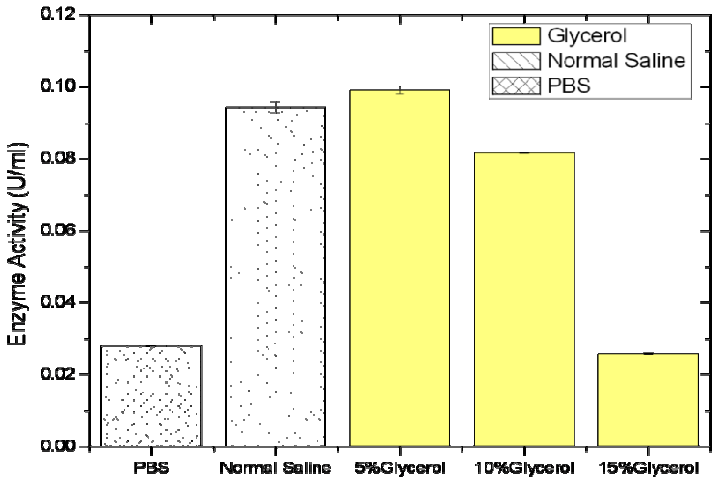


Figure 7D is the graph of GDPH enzyme activity of samples preserved PBS, normal saline and 5%, 10%, 15% glycerol. Sample in 15%glycerol and PBS has shown less enzyme activity in comparison to 5%, 10% glycerol. Samples in 5%glycerol have more enzyme activity than normal saline. However, it is evident from the result that increase in glycerol concentration leads to decrease in enzyme activity indicating the increase in cryoprotectant activity of glycerol.

Figure 7E

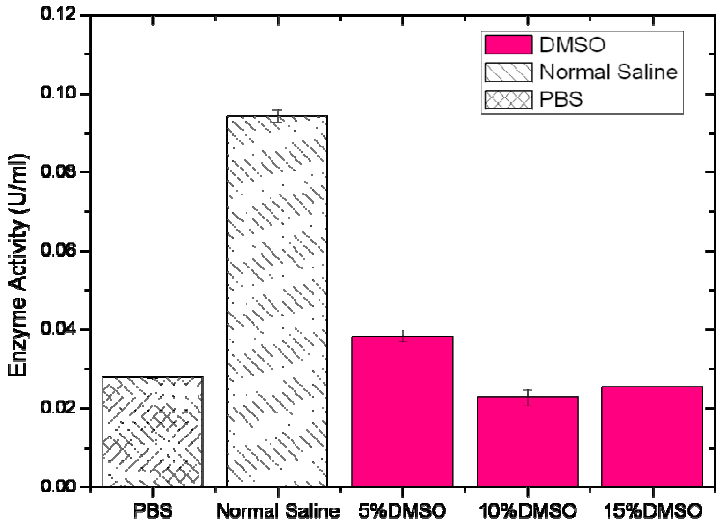


Figure 7E represents the graph of GDPH enzyme activity of samples preserved PBS, normal saline and 5%,10%,15%DMSO. Ten percent DMSO has shown less enzyme activity than 15%DMSO, PBS and 5% DMSO.

Figure 7F

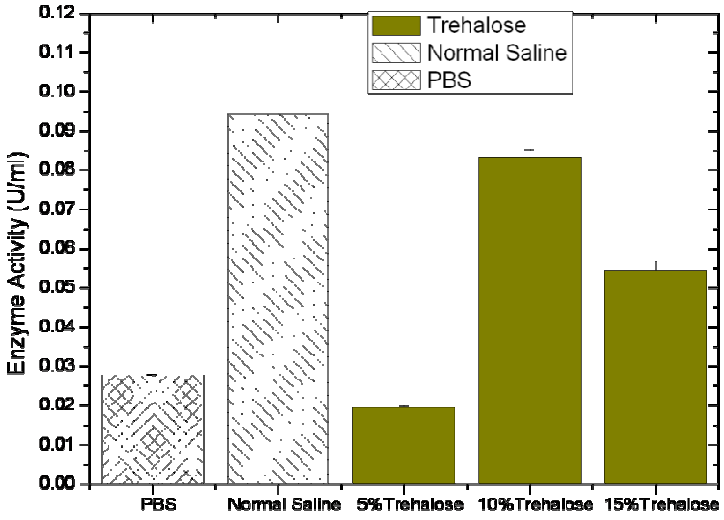


Figure 7F represents the graph of GDPH enzyme activity of samples preserved PBS, normal saline and 5%, 10%, 15% trehalose. Sample in 5%trehalose has shown less enzyme activity than PBS. However, 10% trehalose has shown more enzyme activity than PBS whereas 15% trehalose has shown decrease in enzyme activity than 10% trehalose.

Flow cytometry analysis of Omental derived stem cells at passage 6:

Omental derived stem cells bind with antibodies conjugated with fluorescent dyes are analyzed for the expression mesenchymal stem cell markers.

Figure 8A

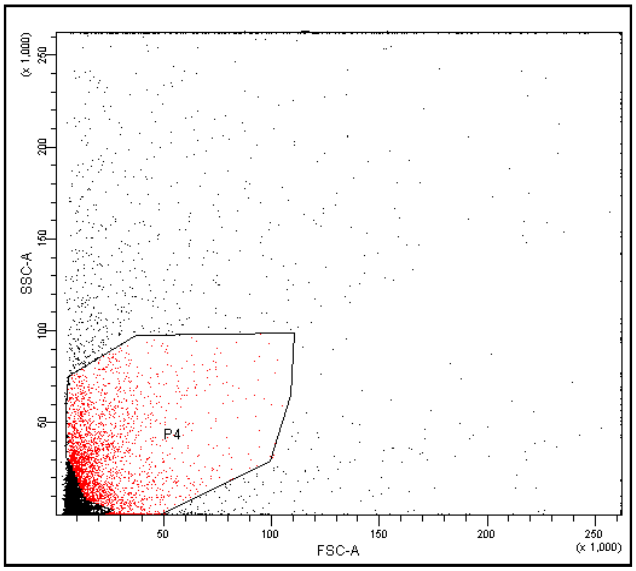


Figure 8A shows forward and side scattering of the cells and the gated region

Figure 8B

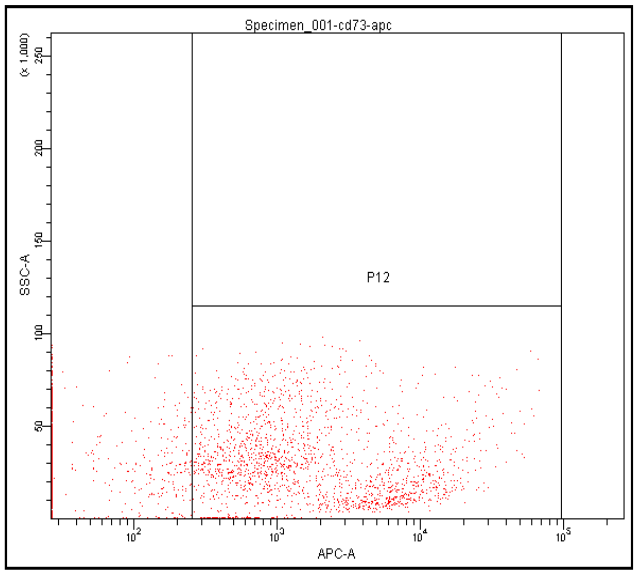


Figure 8B represents the expression level of CD73 that is conjugated with APC-A dye. CD73 has shown 67.2% expression.

Figure 8C

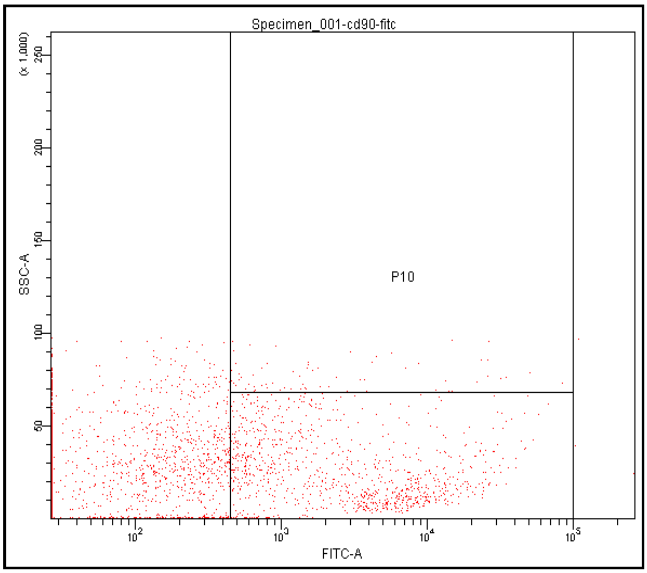


Figure 8C shows the expression level of CD90 that is conjugated with FITC-A dye. CD90 has shown 37.2% expression.

Figure 8D

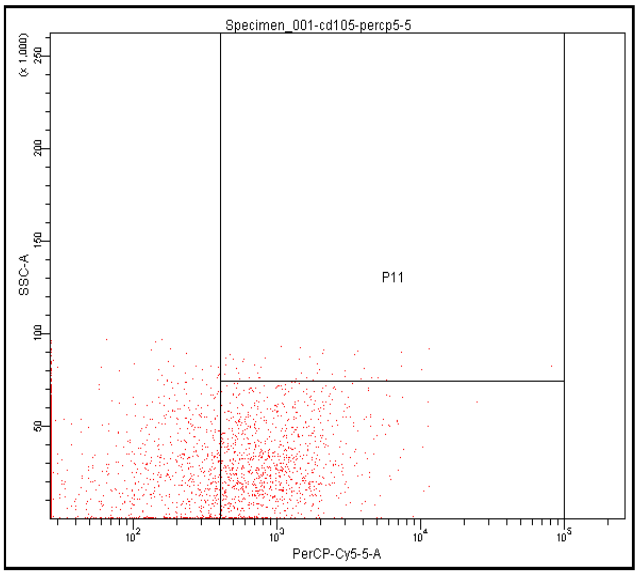


Figure 8D shows the expression level of CD105 that is conjugated with PerCP-Cy5-5-A dye. CD105 has shown 39.9% expression.

Figure 8E

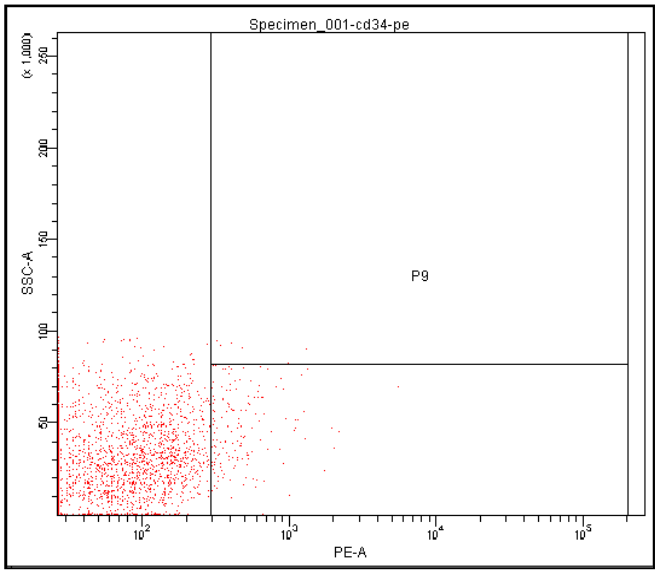


Figure 8E represents the expression level of CD34 that is conjugated with PE-A dye. CD34 has shown 5.1% expression.

Figure 8F

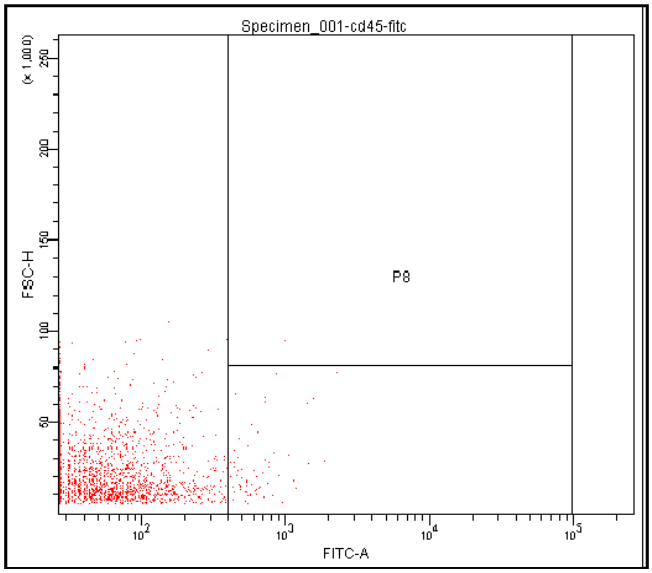


Figure 8F represents the expression level of CD45 that is conjugated with FITC-A dye. CD45 has shown 2.3% expression.

Flow cytometry analysis of Omental derived stem cells at passage 10: Omental derived stem cells bind with antibodies conjugated with fluorescent dyes are analyzed for the expression mesenchymal stem cell markers.

Figure 9A

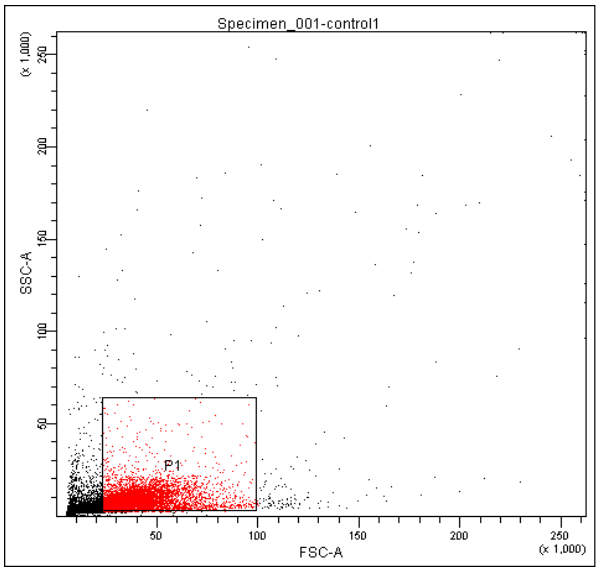


Figure 9A shows forward and side scattering of the cells and the gated region

Figure 9B

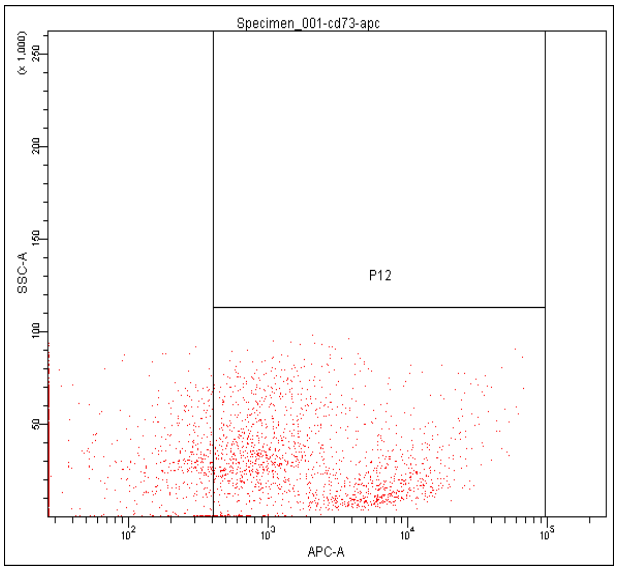


Figure 9B represents the expression level of CD73 that is conjugated with APC-A dye. CD73 has shown 60.2% expression.

Figure 9C

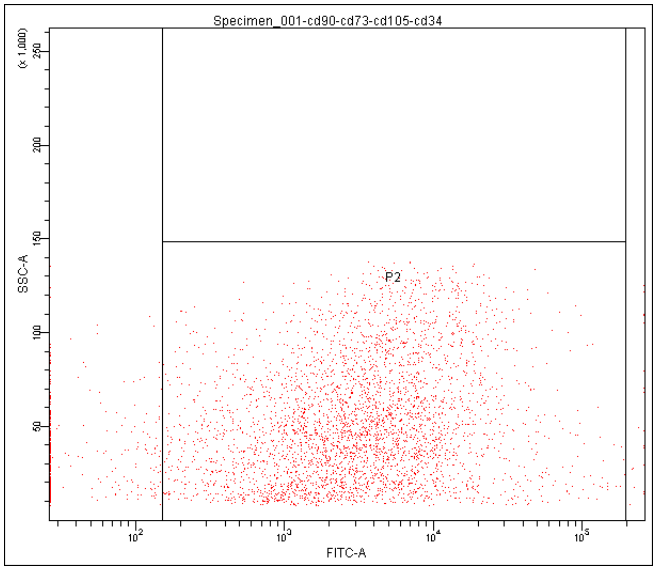


Figure 9C shows the expression level of CD90 that is conjugated with FITC-A dye. CD90 has shown 91.7% expression.

Figure 9D

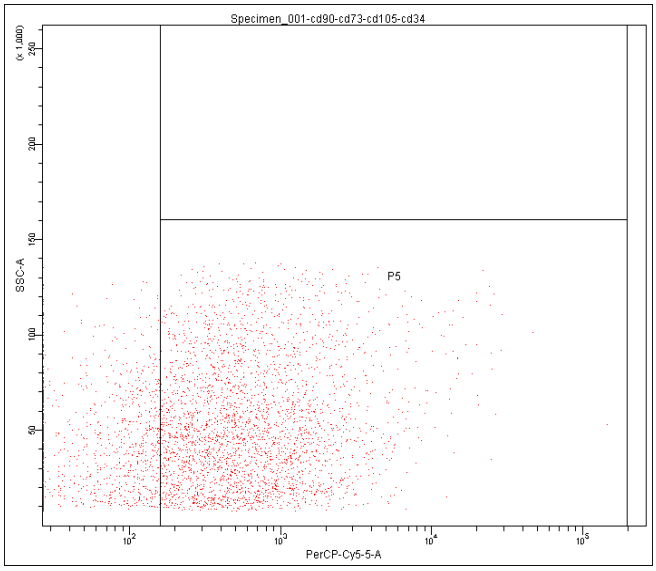


Figure 9D shows the expression level of CD105 that is conjugated with PerCP-Cy5-5-A dye. CD105 has shown 73.2% expression.

Figure 9E

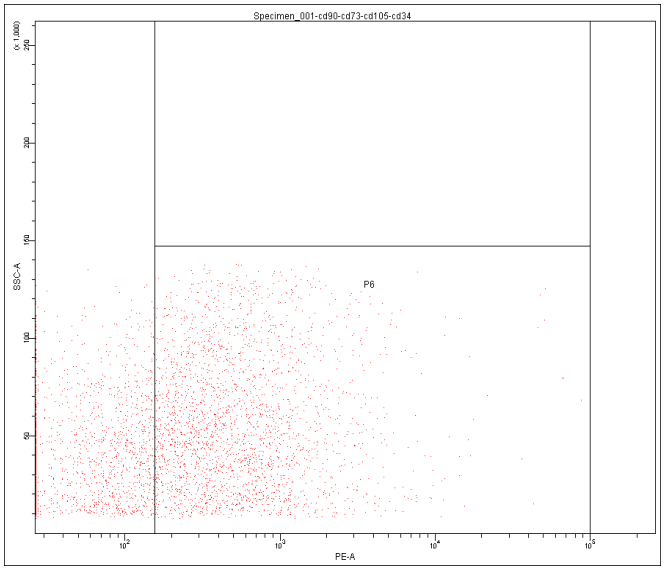


Figure 9E represents the expression level of CD34 that is conjugated with PE-A dye. CD34 has shown 73% expression.

Figure 9F

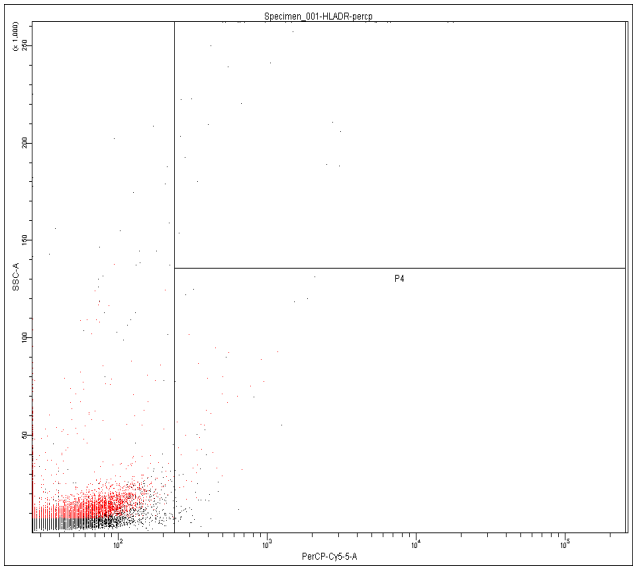


Figure 9F represents the expression level of HLA DR that is conjugated with FITC-A dye. CD45 has shown 1.2% expression.

Adipogenic differentiation:

After 20days of treating the cells with adipogenic induction media, the cells were stained with oil o red stain and observed under phase contrast microscope.

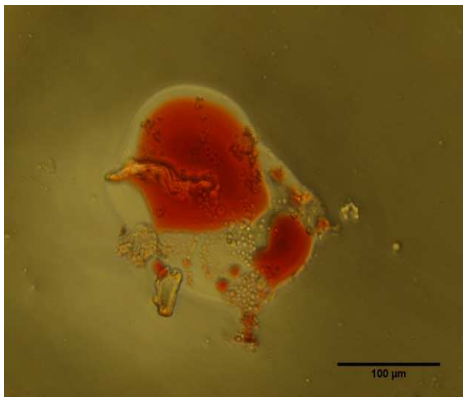


Fig 10A: Oil O red stained cells after adipogenic differentiation (400X magnification)

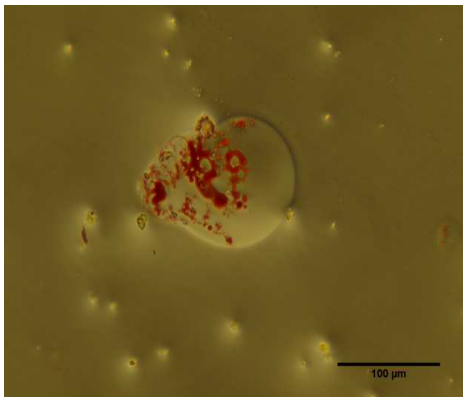


Fig 10B: Oil O red stained cells after adipogenic differentiation (400X magnification)

Osteogenic differentiation:

After 21days of treating the MSCs with osteogenic differentiation, the cells were stained with alizarin red.

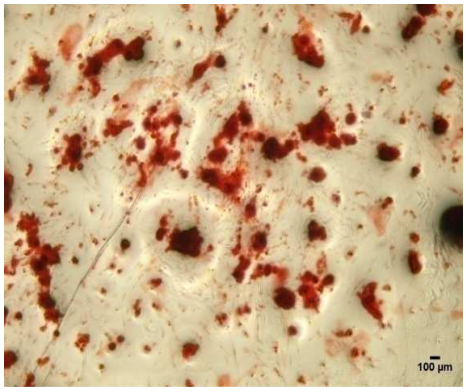


Fig 11A: Alizarin stained cells after osteogenic differentiation (50X magnification)

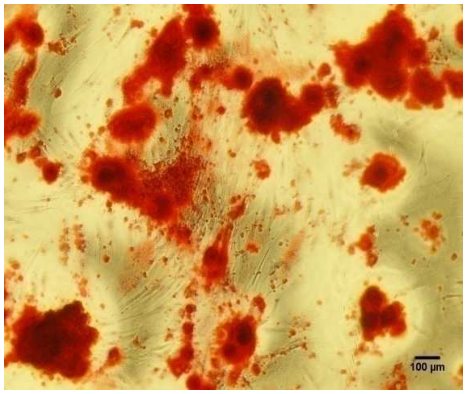


Fig 11B: Alizarin stained cells after osteogenic differentiation (100X magnification)

The 50X magnification images were analyzed by Image J software and the mean percentage of stained area was measured. It was found that **16.43%** area was stained.

Osteogenic differentiation in the presence of 17-β-estradiol:

10nM Concentration

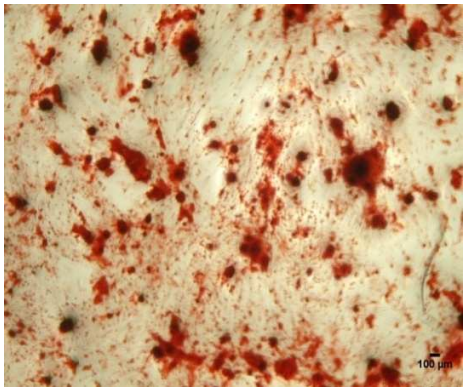


Fig 12A: Alizarin stained cells after osteogenic differentiation in 10nM concentration of 17-β-estradiol (50X magnification)

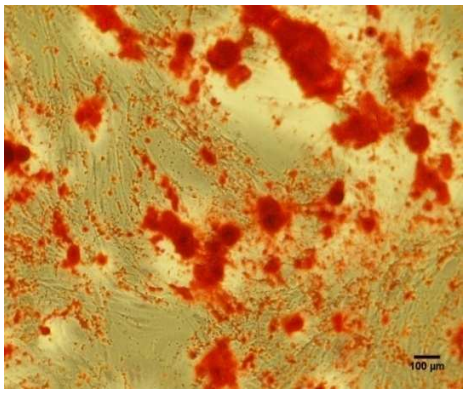


Fig 12B: Alizarin stained cells after osteogenic differentiation 10nM concentration of 17-β-estradiol (100X magnification)

The 50X magnification images were analyzed by Image J software and the mean percentage of stained area was measured. It was found that **22.076%** area was stained in the presence of 10nM 17-β-estradiol

20nM Concentration:

Fig 13A: Alizarin stained cells after osteogenic differentiation in 20nM concentration of 17-β-estradiol (50X magnification)

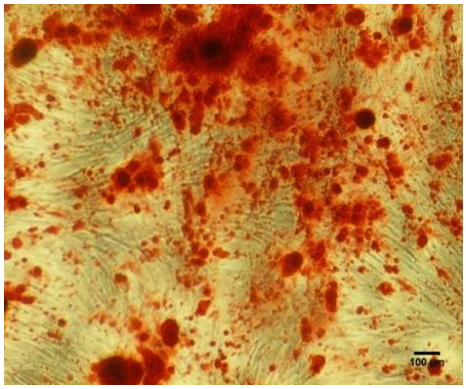
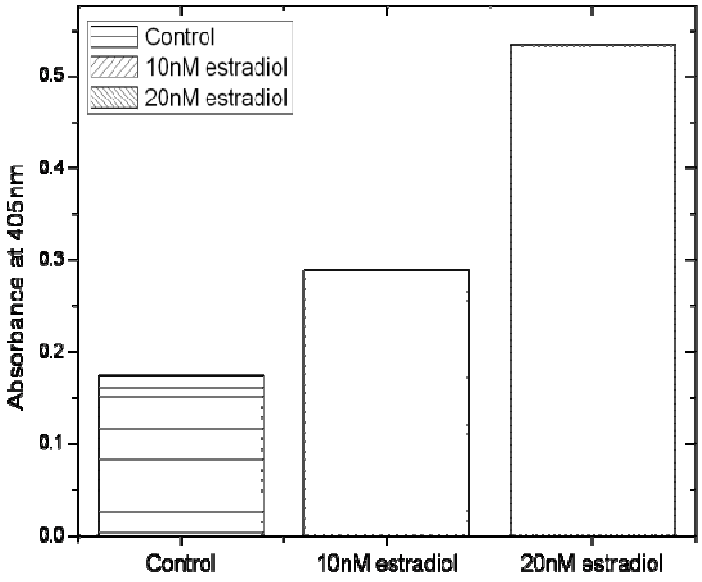


Fig 13B: Alizarin stained cells after osteogenic differentiation in 10nM concentration of 17-β-estradiol (100X magnification)

The 50X images were analyzed by Image J software and the mean percentage of stained area was measured. It was found that **30.488**% area was stained in the presence of 20nM 17-β-estradiol

Quantification of alizarin red stain:

High absorbance of stained alizarin red was recorded in the 20nM estradiol in comparison to 10nM estradiol and control.The control sample, without any presence of estradiol has shown less absorbance.



igure 14: Graph showing the uantification of alizarin red stain

7. Discussion:

Our experiments exposed the cryoprotective nature of Phosphate buffered saline (PBS). PBS has shown good results in comparison to Mannitol, sucrose, glycerol. It is well known that the composition of PBS contains Potassium chloride (kcl), Sodium chloride (Nacl), Di-sodium hydrogen phosphate (Na2HPO4.2H2O), Potassium di-hydrogen phosphate (KH2PO4). Sodium and potassium are intra and extracellular ions that maintain the membrane potential of cell membrane. Cryoinjury caused during freezing leads to the loss of these ions resulting in destabilization of the membrane potential. The presence of sodium and potassium in the PBS may be responsible for the cryoprotective nature of PBS by maintaining the membrane potential. Another possible mechanism will be the potassium ions present in PBS are responsible for synthesizing the cellular currency "ATP". Futher detailed investigations have to be done to prove these assumptions.

The flow cytometric analysis of omental derived stem cells revealed the change in expressions profiles of surface markers from passage to passage. The expression levels of surface markers CD73, CD103, CD90 and CD34 have changed from passage6 to passage10. Interestingly, the expression of CD34 has increased from passage6 to passage10. However, Mitchell et al., reported the decrease in the expression of CD34 as the passage increases. And our result is completely contrary to Mitchell et al., work. The reason might be the using of tissue from different origin. We have isolated the stem cells from omental fat whereas Mitchell and colleagues have worked on stem cells derived from lipoaspirates.

In this study, the effect of 17-β-estradiol has studied osteogenesis of omental fat derived stem cells. The signaling mechanisms involved in the osteogenesis in the presence of 17-β-estradiol are unknown.

8. Conclusion and future work:

The results of the study indicate that Trehalose, DMSO, Glycerol and PBS have shown better cryoprotective action. DMSO and Glycerol are being used from many decades, but in the higher concentrations, these are toxic. Therefore, the ultimate goal will be to replace DMSO and Glycerol with some other ideal cryoprotectants. By the findings of this study, it is proved that PBS can also be used as cryoprotective agent. Further investigation has to be done to determine which component of PBS is having cryoprotective action. The various combinations of individual components of PBS with sugars may help in elucidating the underlying mechanisms of cryoprotective action of PBS.

On the other hand, it was proved that 17-β-estradiol has profound effect on osteogenic differentiation of omental-derived stem cells. The molecular mechanisms behind the effect of 17-β-estradiol has to be determined. The effect of 17-β-estradiol on proliferation rate of omental-derived stem cells from patients of different age and gender has to be studied.

In conclusion, the detailed investigations on the above mentioned studies will help in development of novel advancements in the fields of cryobiology and tissue engineering.

9. References

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